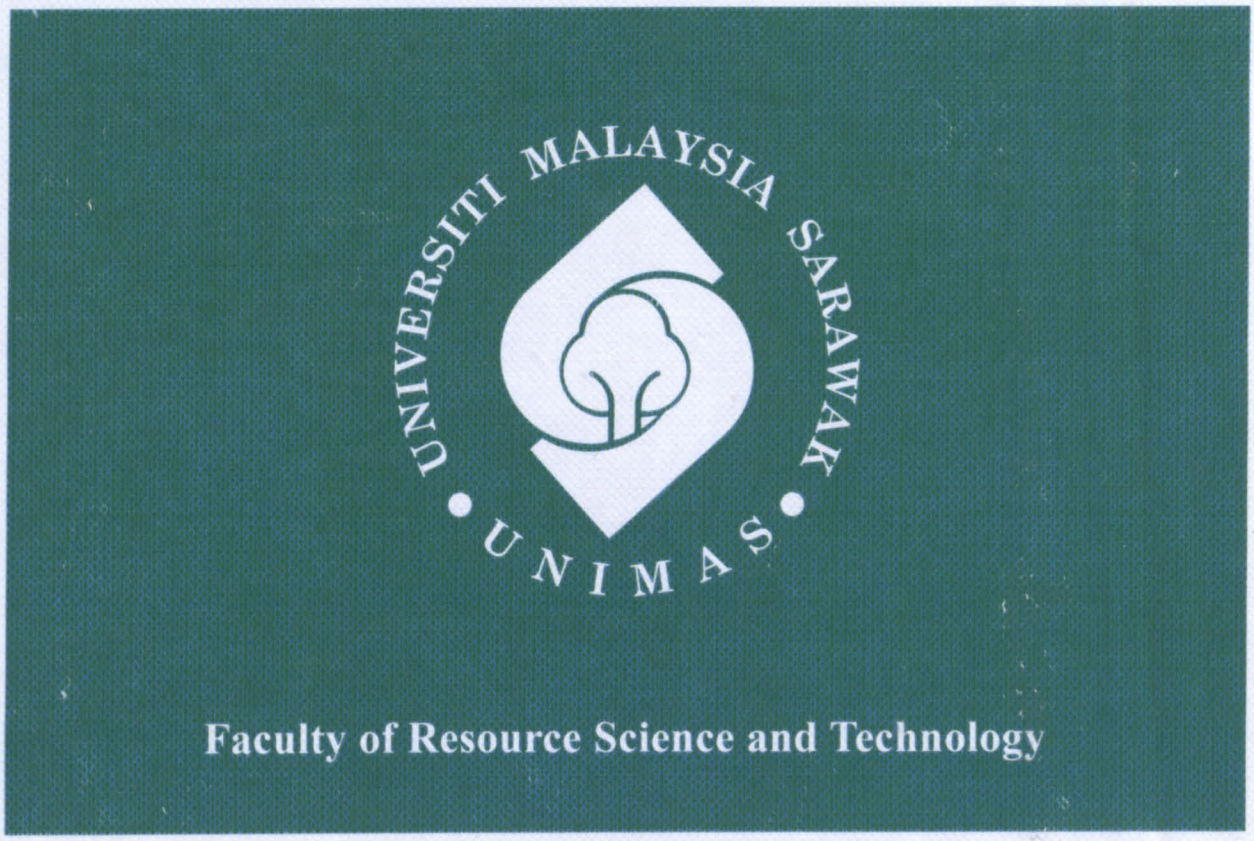

Biofilm Forming Ability and Time Course Analysis of Intermediate *Leptospira* on Biotic and Abiotic Surfaces

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Bachelor of Science with Honours
(Resource Biotechnology)
2017

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Table of Contents

Acknowledgement..... I

Declaration..... II

Table of Contents..... IV

List of Abbreviations..... VI

List of Tables..... VII

List of Figures..... VIII

Abstract..... 1

1.0 Introduction..... 2

2.0 Literature Review..... 5

2.1 *Leptospira sp.*..... 5

2.1.1 Classification of *Leptospira*..... 5

2.1.2 Features of intermediate *Leptospira*..... 6

2.2 Leptospirosis cases..... 6

2.3 Biofilm formation development..... 8

2.4 Time course analysis on biofilm formation..... 9

3.0 Materials and Methods..... 11

3.1 Bacterial strains and culture conditions..... 11

3.2 Dark field microscopic view..... 12

3.3 Biofilm formation on 24-well polystyrene plates 13

3.4 Crystal violet assay 13

3.5 Biofilm formation of intermediate *Leptospira* on wood surface..... 13

3.6 Phase-contrast light microscopy view..... 14

3.7 Data analysis 15

4.0 Results and Discussion 16

4.1 Bacterial strains and culture conditions..... 16

4.2 Dark field microscopic images 16

4.3 Biofilm formation on 24-well polystyrene plates 18

4.4 Biofilm quantification by crystal violet staining 21

4.5 Biofilm formation of intermediate *Leptospira* on wood surfaces..... 25

4.6 Phase-contrast light microscopy visualization..... 32

4.7 Data correlation and clinical implication 37

5.0 Conclusion 40

6.0 References..... 41

List of Abbreviations

EPS	Extracellular Polymeric Substance
EMJH	Ellinghausen McCullough Johnson-Harris
BSA	Bovine Serum Albumin
OD	Optical Density
PBS	Phosphate Buffer Saline

List of Tables

Table	Page
Table 1 - Intermediate *Leptospira* strains obtained from different sources.	12
Table 2 - Optical density values of the selected strains of intermediate Leptospira.	18
Table 3 - The images of the stained well plates which show various colour intensities of intermediate *Leptospira* after aspiration of crystal violet staining from day 1 to 11.	20
Table 4 - Mean OD values of intermediate strains on polystyrene surfaces for 11 days.	21
Table 5 - Optical density values of selected strains of intermediate *Leptospira.*	25
Table 6 - Various colour intensities of intermediate *Leptospira* on wood surfaces after aspiration of crystal violet staining from day 1 to 11.	27
Table 7 - Mean OD values of intermediate strains on wood surfaces for 11 days.	28

List of Figures

Figure	Page
Figure 1 - Dark field microscopic images of intermediate *Leptospira* isolates at 400x magnification.	17
Figure 2 - Biofilm formation by intermediate *Leptospira* on polystyrene surfaces for 11 days.	24
Figure 3 - Biofilm formation by intermediate *Leptospira* on wood surfaces for 11 days.	31
Figure 4 - Light micrographs of *L. wolffii* (CFG14) on glass slides.	34
Figure 5 - Light micrographs of CFG 9 on glass slides.	35
Figure 6 - Light micrographs of CFG 1 on glass slides.	36
Figure 7 – Biofilm formation by CFG 14 on polystyrene and wood surfaces for 11 days	39

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ABSTRACT

Biofilm formation is an essential factor contributing to bacterial pathogenesis. However, limited studies have been documented on biofilm formation of intermediate *Leptospira* on biotic and abiotic surfaces. This study was therefore conducted to analyse the time course and the biofilm forming ability of intermediate *Leptospira* on 24-well polystyrene plate surface and wood surface. Quantified biofilms of 6 intermediate *Leptospira* strains were carried out on polystyrene and wood surfaces using crystal violet binding assay to assess the biofilm forming abilities based on optical density obtained. A significant difference ($p<0.05$) exists in biofilms produced by *Leptospira* on wood surface which formed more biofilm than on polystyrene surface. In this study, the strongest biofilm producer is CFG14 with OD_{600} of 2.283 on day 8 and OD_{600} of 2.333 on day 6 for polystyrene and wood surface, respectively. Visualisation of biofilm by phase-contrast microscopy of two representative strains correlated with the OD values obtained and the colour intensity of stained well plates and wood surfaces. Overall, all of the intermediate *Leptospira* were able to form biofilm which contributed to survival in diverse environment and disease transmission. These results provided better understanding on contact time of biofilm formation by intermediate *Leptospira* and can assist the authority such as Ministry of Health and the medical practitioners in devising an appropriate control strategy to reduce transmission of leptospirosis.

Key words: *Leptospira*, biofilm formation, crystal violet assay, contact time

ABSTRAK

Pembentukan biofilm ialah faktor penting yang menyumbang kepada patogenesis bakteria. Walaubagaimanapun, kajian yang dilakukan tentang pembentukan biofilm Leptospira perantaraan pada permukaan biotik dan abiotik adalah terhad. Justeru, kajian ini dijalankan untuk menganalisa tempoh masa dan kebolehan membentuk biofilm Leptospira perantaraan pada 24-plat berlubang dan permukaan kayu. Kuantifikasi biofilm 6 jenis Leptospira perantaraan telah dilakukan di atas permukaan 24-plat berlubang dan permukaan kayu dengan menggunakan protokol ungu kristal untuk menganalisa kebolehan membentuk biofilm berdasarkan nilai ketumpatan optik. Perbezaan signifikan ($p<0.05$) wujud pada biofilm yang dibentuk Leptospira perantaraan pada permukaan kayu yang mencatat nilai lebih tinggi berbanding dengan permukaan 24-plat berlubang. Dalam kajian ini, CFG 14 ialah pembentuk biofilm tertinggi dengan nilai ketumpatan 2.283 pada hari kelapan atas permukaan 24-plat berlubang dan nilai ketumpatan 2.333 pada hari keenam atas permukaan kayu. Pengimejan biofilm menggunakan mikroskop fasa-kontras yang diwakili dua jenis Leptospira perantaraan mempunyai hubungkait dengan nilai ketumpatan optik dan keamatan warna di 24-plat berlubang dan permukaan kayu. Secara keseluruhan, kesemua jenis Leptospira perantaraan dapat membentuk biofilm yang menyumbang kepada survival bakteria di pelbagai habitat dan transmisi penyakit. Pengetahuan semasa daripada kajian ini dapat meningkatkan pemahaman tentang masa sentuhan pembentukan biofilm oleh Leptospira perantaraan dan dapat membantu pihak berkuasa seperti pihak Kementerian Kesihatan dan pengamal perubatan dalam merangka strategi kawalan yang sesuai bagi mengurangkan transmisi leptospirosis.

Kata kunci: *Leptospira, pembentukan biofilm, protokol ungu kristal, masa sentuhan*

1.0 Introduction

Leptospirosis is a global zoonotic disease which is caused by *Leptospira* sp. It is transmitted through contact with urine, water, or soil contaminated by urine from animal reservoirs, such as rodents, dogs, and livestock (Guerra, 2009). Direct penetration of L*eptospira* sp. through the conjunctiva or surface epithelium could also cause the transmission to occur (Russ *et al.,* 2003). *Leptospira* sp. can be divided into three types which are saprophytic, intermediate, and pathogenic (Ristow *et al.,* 2008). In Malaysia, the Prevention and Control of Infectious Diseases Act 1988 has officially published leptospirosis as a notifiable disease in 2010. There were 616 cases and 24 deaths resulted from leptospirosis reported in 2014 meanwhile, a total of 163 cases with six deaths were reported on 7th March 2015 (Sarawak Health Department, 2015). The factors which contribute to endemic leptospirosis include tropical climates, exposure of mammalian reservoirs to human populations and low level of sanitary in the environment (Levett, 2001). According to Thayaparan *et al.* (2013), a leptospirosis occurrence among mammals (squirrels, bats, primates, mongoose and rats) was reported in the wildlife areas in Kuching, Sarawak. Furthermore, according to Pui *et al.* (2015), there was a distribution of *Leptospira* sp. in the national parks of Sarawak which include Bako National Park and Tanjung Datu National Park.

The presence of *Leptospira* sp. posed the risk of transmission and infection to humans. The survival of *Leptospira* sp. is enhanced through the biofilm formation as they are able to shield themselves from external stress (Kumar, 2015). According to Kreth *et al.* (2004), the characteristics of biofilm can be described by various parameters such as cell mass, adhesive strength and architecture. These parameters determine the physiological states of the constituent cells and affect the effectiveness of mechanical removal of

biofilms from surfaces. The cells growing in biofilms will develop increased resistance to antibiotics as compared with their free-living counterparts (Costerton *et al.*, 1999). Since biofilm formation plays an important role in areas from environmental industry to medicine, understanding the mechanisms of biofilm formation has become the focus of the biofilm research community.

The time course study of biofilm formation is essential in understanding the contact time required for the attachment of *Leptospira* on a surface. Moreover, the duration taken for the biofilm formation by *Leptospira* is also significant to fully understand its level of pathogenicity. It had been proven that, in other organisms, such as *Salmonella* Typhi, the biofilm formation can be a factor which contributes to cross contamination. As the time increases, the biofilm is tough to be removed during the rinsing process (Tang *et al.*, 2012).

Biofilm formation is an essential factor contributing to bacterial pathogenesis and disease control. However, limited studies have been documented on biofilm formation of Intermediate *Leptospira* on biotic and abiotic surfaces. This study was therefore conducted to analyse the biofilm forming ability and the time course of intermediate *Leptospira* on 24-well polystyrene plate surface and wood surface.

It is hypothesised that biofilm formation rate of intermediate *Leptospira* is higher on wood than polystyrene because of higher level of surface roughness.

Thus, this research is carried out to address the following research problems:

1. Are the selected intermediate *Leptospira* able to form higher biofilm formation rate on wood surface than polystyrene surface?
2. Are the selected intermediate *Leptospira* able to form biofilm on abiotic and biotic surfaces?

3. What is the relationship between contact time and formation of biofilm by the selected intermediate *Leptospira*?
4. What is the evaluation of biofilm formation by the selected intermediate *Leptospira* in terms of quantity (mass) and quality (adhesive strength)?

The objectives of this study are:

1. To determine the ability of the selected intermediate *Leptospira* to form higher biofilm formation rate on wood surface than polystyrene surface.
2. To determine the biofilm formation ability by selected intermediate *Leptospira* on abiotic and biotic surfaces.
3. To determine the relationship between contact time and biofilm formation of selected intermediate *Leptospira.*
4. To evaluate the biofilm formation by selected intermediate *Leptospira* in terms of quantity (mass) and quality (adhesive strength).

2.0 Literature Review

2.1 *Leptospira sp.*

2.1.1 Classification of *Leptospira*

Leptospira originates from the Greek word, *'Leptos'*, which is defined as coiled, and spiral that describe the morphological structures of *Leptospira* (Dutta & Christopher, 2005). These bacteria could survive under favourable condition at optimum temperature of 28°C to 30°C, hence, they are considered as obligate aerobes (Thayaparan *et al.*, 2013). According to Haake (2000), *Leptospira* is a spirochete that has both features of Gram-positive and Gram-negative bacteria. *Leptospira* sp. can be divided into three types which are saprophytic, intermediate, and pathogenic (Ristow *et al.*, 2008).

The *Leptospira* genus is classified serologically into two species, which include *Leptospira interrogans* and *Leptospira biflexa. Leptospira interrogans* consist of all pathogenic strains and *Leptospira biflexa* comprise of all saprophytic strains (Evangelista & Coburn, 2010). According to Mayer-Scholl *et al.* (2014), the genotypic classification has defined 20 species within *Leptospira* genus. The classification which is based on DNA hybridization studies or 16S rRNA gene sequencing had clustered the species into saprophytic, pathogenic and intermediate *Leptospira. Leptospira* are classified into serovars by the expression of epitopes in a mosaic of the lipopolysaccharides (LPS) antigens. The antigenically related serovars can be classified into serogroups (Mohammed *et al.*, 2011).

2.1.2 Features of intermediate *Leptospira*

Intermediate *Leptospira* is a group of *Leptospira* species that are closely related to pathogenic and saprophytic *Leptospira*. Ko *et al.* (2009) stated that this species still has unclear pathogenicity, thus it can be considered as an opportunistic bacterium. Phylogenetic studies had been carried out on *Leptospira* strain Bairam-Ali and the results shown that this strain has both characteristics of pathogenic and saprophytic *Leptospira* (Voronina *et al.*, 2014). This unpredicted finding of intermediate *Leptospira* has brought essential information especially on the ability of the bacteria to adapt to different environmental condition. Intermediate *Leptospira* also has the ability to cause disease in animals and human, although the cases are less frequent (Ricaldi *et al.*, 2012).

Since little is known about the pathogenicity of intermediate *Leptospira* group (Slack *et al.*, 2006), it is postulated that *Leptospira wolffii* serovar Khorat strain Khorat-H2 has the ability to evolve into life-threatening complications. Therefore, the detection of this intermediate *Leptospira* strain from the environment indicated that precautionary action should be taken in handling the soil and water samples at study areas (Pui *et al.*, 2015).

Thus, more studies need to be done on intermediate *Leptospira* in order to investigate its transmission of pathogenicity.

2.2 Leptospirosis cases

Leptospirosis is an endemic disease in Southeast Asia, Latin America, Oceania, Caribbean, and the Indian subcontinent (Evangelista & Coburn, 2010). According to Thayaparan *et al.* (2013), these cases are often reported in tropical climates country because of the ability of *Leptospira* to survive and live in a wide range of animal species

especially domestic animals and human. Furthermore, the high incidence of leptospirosis is due to the ability of *Leptospira* to survive outside the host as long as the surroundings provide favourable conditions. Based on the study carried out by Tan (1970), leptospirosis is an occupational disease. General labourers and rubber estate workers were the main targets due to their regular exposure to the environmental sources (Omar, 1967). Besides, the potential areas for the spread of leptospirosis are usually located at the lake, stream, and pond where recreational activities took place (Victoriano *et al.,* 2009). The isolation of *Leptospira interrogans* serovar Hebdomadis from water and soil samples in National Service Training Centres in Kelantan and Terengganu, Malaysia by Ridzlan *et al.* (2010) had gained public concern.

Balamurugan *et al.* (2013) have conducted a research in India and found out that there is high number of leptospirosis cases were reported. The cases are difficult to be controlled due to poor sanitation and climate factors. Vitale *et al.* (2005) also stated that 300 leptospirosis cases occurred annually in Brazil and the number of cases is increasing over time. Similarly, the incidence rates of leptospirosis in Malaysia is 2 to 5 of 100 000 population (Lim *et al.*, 2011). Seasonal rainfall in Malaysia contributes to the increment of leptospirosis cases. Sarawak also recorded high number of cases over time (Sarawak Health Department, 2015).

A leptospirosis occurrence among various mammals which include mongoose, rats, bats, primates and squirrels was reported in the wildlife regions in Kuching, Sarawak (Thayaparan *et al.,* 2013). In addition, it had also been confirmed that there was a distribution of *Leptospira* sp. in Bako National Park and Tanjung Datu National Park which are located in Sarawak (Pui *et al.,* 2015).

2.3 Biofilm formation development

According to Costerton (1999), bacterial biofilm is defined as an organized community of bacterial cells which is enclosed in a self-produced polymeric matrix and stick to an inert or living surface.

Bacterial biofilms have a structurally dynamic and complex architecture and can form on various surfaces. The development of biofilm formation by *Leptospira* mimics other biofilm described in the literature, beginning with individual bacteria adhering to the surface, then the expansion of the bacteria into micro colonies, and the formation of a three dimensional structure. Bacteria in biofilms have properties distinct from planktonic cells, such as increased resistance to biocides and antimicrobial agents (Hall-Stoodley *et al.*, 2004).

Biofilm formation begins with the adhesion of planktonic bacteria to surfaces, including abiotic surfaces (plastics, glasses, metals and minerals) and biotic surfaces (plant and animal tissues). The first colony that sticks to a surface is normally weak and the adherence is reversible through Van der Waals forces. The next colony will be provided with additional adhesion sites by the first colony. The attached cells then will produce a matrix, consisting of extracellular polymeric substance (EPS), and multiply, thereby forming a microcolony. From this microcolony, a mature biofilm can arise. EPS is also referred to as slime. In the final stage of biofilm formation, cells may detach and return to planktonic life or die (Hall-Stoodley *et al.*, 2004). Hydrophobicity of the bacteria and the attachment surface are the essential measures in biofilm formation. The microbial community communicate within themselves by using cell signalling molecules or known as Quorum Sensing phenomenon. Chemical messages are emitted by the bacteria in Quorum Sensing phenomenon (Deb *et al.*, 2014).

According to Ganoza *et al.* (2006), it was recently revealed that severe leptospirosis was linked with exposure to a high concentration of leptospires in environmental water samples. This analysis shows that the biofilm formation may contribute to long-term survival of *Leptospira* in environmental water (Trueba *et al.*, 2004).

2.4 Time course analysis on biofilm formation

Time course analysis is a study which observes varying activities of a mechanism over time. Time course study of biofilm formation is carried out to observe the relationship between contact time and the formation of biofilm. According to Tang *et al.* (2012), the biofilm forming ability by *Salmonella* Typhi varies with increment of time. The OD value which represents the quantity of biofilm is 0 at 0 h because the cells need to adapt to the new environment condition when the bacterial cells are transferred onto a new surface. The bacterial cells have enough time to attach to the surface as the contact time increases. Thus, the OD values increase with incubation time. This indicates that the interaction forces between the surface and biofilm become stronger (Ukuku & Fett, 2002).

Biofilm formation is considered a selective advantage for staphylococci mastitis isolates, facilitating bacterial persistence in the udder. It requires attachment to mammary epithelium, proliferation and accumulation of cells in multilayers and enclosing in a polymeric matrix, being regulated by several loci. As biofilm formation can proceed through different pathways and time ranges, its detection may differ according to the time of observation.

According to Oliveira *et al.* (2007), the biofilm-forming ability increased with incubation time for *Staphylococcus aureus* and *Staphylococcus epidermidis* by Fluorescent In Situ Hybridisation (FISH). The isolates were able to produce biofilm at 24, 48 and 72 h.

The biofilm formation is measured in terms of quantity and quality for every subsequent interval of time, thus the changes in the formation of biofilm can be observed. The stages that the bacteria go through in order to form a strong slime is studied by observing and recording the changes in the formation of the biofilm.

3.0 Materials and Methods

3.1 Bacterial strains and culture conditions

The strains of intermediate *Leptospira* were obtained from the Microbiology Laboratory stock culture collection, Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak. The six selected intermediate strains were collected from different sources such as soil, water and rat liver by previous researchers. The strains were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid media (Difco, Becton-Dickinson, USA) in which 0.1g of 5-fluoroacil were added to reduce contamination. Leptospires require fatty acids as the main source of energy and carbon which are used as a source of cellular lipids, however, leptospires are unable to synthesize fatty acids de novo. Thus, EMJH medium were used to cultivate the leptospires because it contains vitamin B1, vitamin B12 and long chain fatty acids (Tween 80) (Nagarajan *et al.,* 2015). The cultures were incubated at room temperature for 30 days. EMJH broth was used as the negative control. The selected intermediate *Leptospira* strains are as shown in Table 1.

Table 1. Intermediate *Leptospira* strains which were obtained from different sources

Isolates strain	***Leptospira* lab no. designation**	**Sources**	**Locality**
Leptospira wolffii serovar Khorat strain Khorat-H2	CFG12	Soil	Miri National Service Training Centre
Leptospira wolffii serovar Khorat strain Khorat-H2	CFG14	Soil	Kubah National Park
Leptospira sp. MS341	CFG9	Water	Miri National Service Training Centre
Leptospira wolffii serovar Khorat strain Khorat-H2	CFG11	Water	Miri National Service Training Centre
Leptospira wolffii serovar Khorat strain Khorat-H2	CFG29	Rat liver	Kampung Sungai Mata
Leptospira wolffii serovar Khorat strain Khorat-H2	CFG1	Soil	POSITIVE CONTROL

3.2 Dark field microscopic view

The isolates were pipetted out (0.1 µl) and transferred on sterile glass microscope slides (25.4 x 76.2 mm, SAIL BRAND, China) after 30 days of incubation. Then, their viabilities were viewed using the dark field microscope, Olympus Compound Microscope BX51 (Olympus Corporation, Japan) under 400x magnification. The images of the intermediate *Leptospira* strains were captured for analysis. The reason why dark field microscope was chosen to observe the bacterial strains instead of the light field microscope is because it is very effective in showing the details of translucent, live and unstained samples. However, one of the disadvantages of dark field microscope is that it provides limited colours, thus the results will show black and white images.

3.3 Biofilm formation on 24 well-polystyrene plates

The method is based on Lambert *et al.* (2012). The spectrophotometer (Metertech Inc.) was used to measure the optical density of the *Leptospira* population at 420 nm before the biofilm formation begins (Sutton, 2006). This helps to ensure the readings of the isolates to be in the range of 0.3 to 0.4 which correspond to 10^6 to 10^8 CFU/ml, indicating the mid-exponential phase of the culture (Lourdat *et al.*, 2009). One ml of every isolates was dispensed into the wells of Tissue Culture Test Plate 24 (TPP, Switzerland). Every isolates analysis were performed in duplicates.

3.4 Crystal violet assay

The crystal violet staining method is based on Ristow *et al.* (2008). It was carried out for every 24 hours. The cultures were dispensed out of the well and rinsed with 1 ml distilled water. The wells were air dried for 15 minutes in room condition. The wells then were fixed with 1 ml of 2% sodium acetate for 5 minutes. Next, the sodium acetate was aspirated out from the well and it was set to be air dried for 15 minutes again. Then, 0.1% of crystal violet was aspirated into every well and set to stain for 20 minutes. Finally, crystal violet was pipetted out and the wells were rinsed with distilled water for three times. Crystal violet solution is used because it functions in staining the cells and the polysaccharide component in biofilm layer. Ethanol/acetone (80/20) was used to solubilize the crystal violet staining at the end of the experiment. The optical density of every isolates was measured by using spectrophotometer (Metertech Inc.) at 600 nm.

3.5 Biofilm formation on wood surface

The biofilm formation on wood surface was determined as described by Stepanovic et al. (2004). A wood block (*Jelutong Paya* Hardwood) was cut by using a small saw into 154 chips measuring 1cm x 1cm x 0.5cm. The chips were washed with detergent (Unilever, Breeze, Malaysia), rinsed with sterile distilled water and air-dried before being

placed into hot air oven at 75°C for 30 minutes. Then all of the wood chips were incubated in the intermediate *Leptospira* isolates for intervals up to 11 days. At the end of each incubation period, the chips were aseptically removed from the culture for biofilm quantification using the crystal violet binding assay described by Stepanovic *et al.* (2004). The chips were washed with 1 ml of Phosphate Buffer Saline (PBS) to remove non-adherent cells. PBS is used because it is isotonic and maintains constant physiological pH. Besides, PBS is reliable and accurate to show consistent removal of unstained crystal violet when performing washing step. In addition, PBS gives low difference of salinity level by reducing the chances of cell bursting followed by cell death (Das & Dash, 2015). Then, the remaining adhered cells were fixed with 2.5 ml of methanol for five minutes. Methanol is used to fix the surface-associated cells. Methanol fixation gives more reliable staining data compared to other fixation method (Minnerath *et al.,* 2009). This is due to the fact that bacterial cells fixed with methanol are able to retain the crystal violet stain more readily and helps the cells to adhere more effectively on surfaces. Each chips were then stained with 0.9 ml of 0.1 % crystal violet for 15 minutes. The excess stain was washed with 1 ml of PBS for three times. After the chips were air-dried, the dye bound to adherent cells were re-solubilised with 2.5 ml of 33% (v/v) glacial acetic acid. The re-solubilised liquid for each chip were poured into cuvette for measurement of absorbance (optical density) using spectrophotometer (Metertech Inc.).

3.6 Phase-contract microscopy view

The procedure was carried out according to Ristow *et al.* (2008). Glass slides were incubated half submerged in the bacterial suspension with 10^6 to 10^8 concentrations. Then, the biofilm formation were observed at different intervals (1, 6, 24, 48, 96, 144, 168, 216, 264 hours). At each interval, the glass slides were rinsed three times in distilled water, air-dried and fixed by heating. Each glass slides were observed by phase contrast microscopy

using Olympus Inverted IX51 microscope (Olympus Corporation, Japan) at 600x magnification.

3.7 Data analysis

The biofilm quantification data obtained from the project were statistically analysed. The quantification of biofilm formation were analysed by the mean optical density of the duplicate readings. Based on the data obtained, graphs were constructed in Microsoft Excel 2010. Then, error bars were also constructed to denote the standard errors for the mean of duplicate readings. Statistical analysis was performed by using Microsoft Excel 2010. Differences between means for the isolates were evaluated using one way ANOVA. The level of significance was set at $p<0.05$.